How a first research experience had an impact on my scientific journey

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ABSTRACT **As I look back to my scientific trajectory on the occasion of being the recipient of the E. B. Wilson Medal of the American Society for Cell Biology, I realize how much an early scientific experience had an impact on my research many years later. The major influence that the first scientific encounters can have in defining a scientist's path makes the choice of the training environment so important for a future career.**

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During my scientific career I have worked on different topics. However, my scientific journey represents a progression of steps that build onto each other. Each experience, no matter how successful, had an impact on how I interpreted subsequent results, on how I assessed their implications, and on how I made subsequent programmatic decisions. My research at the interface of cell biology and neuroscience has spanned from membrane traffic and membrane remodeling, to phosphoinositide signaling, and more recently also to the role of membrane contact sites in the control of membrane lipid homeostasis in physiology and disease. While only partially related, all these topics are interconnected by an intellectual thread. As an example—which shows how defining our training years can be—I will summarize here how an early scientific experience in medical school contributed to shape in unexpected ways my research directions many years later.

From the day I enrolled in medical school at the University of Milano I knew I wanted to be a scientist. While a medical student, I explored several research environments that would expose me to leading-edge science. Eventually I encountered a young scientist, Jacopo Meldolesi, who had just returned to Italy after studying the secretory pathway as a postdoc with Jim Jamieson and George Palade at Rockefeller University, and I decided to join his lab to carry out the required short medical school thesis project. Jacopo was interested in the regulation of secretion and proposed that I explore mechanisms underlying the so-called "PI (Phosphatidyl Inositol) effect" described by Hokin and Hokin in the 1950s (Hokin and Hokin, 1953). These investigators had found that stimulation of secretory cells resulted in the rapid cleavage of PI immediately followed by its resynthesis. It remained unclear whether these changes reflected a signaling reaction elicited by secretagogues or metabolic changes associated with membrane traffic reactions triggered by the stimulus. So, the idea was to determine in which membrane compartment this turnover occurred: selectively at the plasma membrane (thus supporting a role in signaling) or in membranes of the secretory pathway. We were puzzled by finding that at least under the rather poor time resolution of our experiments—incubation of pancreatic lobules for tens of minutes with radioactive inositol following stimulation with acetylcholine—newly synthesized PI was found at similar concentrations in all membranes. But then we became aware of the recently described proteins that transport lipids between membranes through the cytosol and independent of membrane traffic (Wirtz and Zilversmit, 1969). We thought that perhaps these proteins could contribute to rapidly equilibrating PI pools in different compartments, questioning the validity of our experimental approach to identify the site of PI resynthesis. I moved on to other projects, and I thought I would never again work with lipids.

In the following years the work of several labs established that the PI effect discovered by Hokin and Hokin mainly reflected agonist-stimulated phospholipase C (PLC)-dependent cleavage of the phosphoinositide $PI(4,5)P_2$ at the plasma membrane to generate the second messengers diacylglycerol (DAG) and IP3 (phosphoinositides are the phosphorylated products of PI) (Lapetina and Michell, 1973; Inoue *et al.*, 1977; Berridge *et al.*, 1983). These findings became textbook knowledge, and the idea that $PI(4,5)P_2$ had primarily a function in signal transduction at the cell surface became mainstream. The additional discovery of the $PI(3,4)P_2$ and $PI(3,4,5)P_3$ as a mediators of growth factor actions added a new twist to the field by showing that not only metabolites generated by $PI(4,5)P_2$ cleavage, but also phosphoinositides themselves, could have a signaling role (Traynor-Kaplan *et al.*, 1988; Whitman *et al.*, 1988; Auger *et al.*, 1989).

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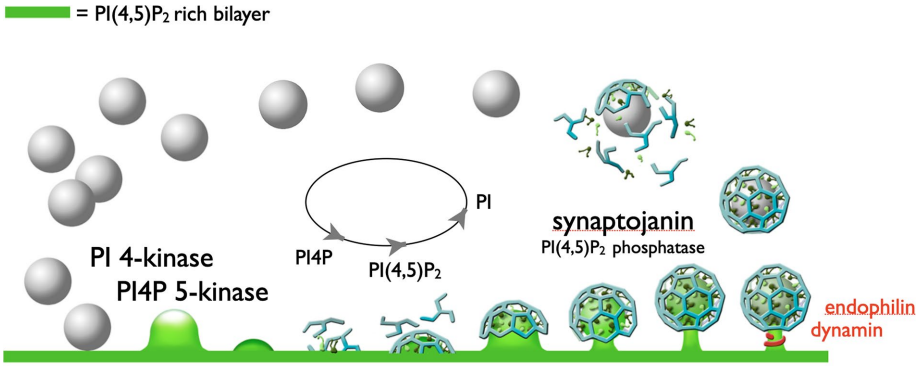

FIGURE 1: Schematic cartoon depicting the occurrence of a cycle of phosphatidylinositol phosphorylation–dephosphorylation nested within the exo-endocytic recycling of synaptic vesicles at synapses. $PI(4,5)P_2$ in the plasma membrane helps define this membrane as the acceptor for the fusion of synaptic vesicles and functions as a coreceptor for endocytic factors responsible for their reinternalization. $PI(4,5)P_2$ dephosphorylation by synaptojanin allows the shedding of endocytic factors and the reutilization of vesicles for a new cycle of secretion. Recruitment of synaptojanin is coupled to dynamin-dependent fission via its binding to the BAR domain–containing protein endophilin, a curvature-generating/sensing protein that also binds dynamin.

However, once again, this signaling appeared to be implicated in classical signal transduction, not in membrane traffic.

In the meantime, I had moved to Yale, first as a postdoc with Paul Greengard and then as a faculty in the Section of Cell Biology (which subsequently became the Department of Cell Biology). Following my postdoc with Greengard, I had become interested in the cell biology of synapses and in the molecular machinery underlying the exo-endocytic recycling of synaptic vesicles. In what turned out to be a seminal finding, in the mid-1990s Peter McPherson, a postdoc in my laboratory, identified a nerve terminal–enriched protein that shared some interactions with the GTPase dynamin, another abundant presynaptic protein studied in our lab (McPherson *et al.*, 1994). As dynamin mediates the fission reaction of endocytosis by cutting the neck of endocytic buds to generate free vesicles (Takei *et al.*, 1995; Roux *et al.*, 2006; Ferguson and De Camilli, 2012), we speculated that this new protein would also have a role in endocytic traffic. Peter set out to clone it, and I vividly recall my surprise when, upon my returning from a summer vacation in Italy he reported to me that a domain of this protein, which we called synaptojanin, had homology to an inositol 5-phosphatase that dephosphorylates $PI(4,5)P_2$ (McPherson *et al.*, 1996). A second domain of this protein was subsequently found to have inositol 4-phosphatase activity (Guo *et al.*, 1999), so that synaptojanin can dephosphorylate $PI(4,5)P_2$ all the way to PI. Thus, I was back to lipids, specifically to inositol phospholipids, and to a potential role of these lipids not in the transduction of extracellular signals, but in membrane traffic. With a mind primed by my project in medical school, I delved with enthusiasm into this topic. $PI(4,5)P_2$ had been shown to interact with clathrin adaptors (Beck and Keen, 1991), but the physiological significance of these findings had remained unclear. Our studies led to a model in which the selective concentration of $PI(4,5)P_2$ in the plasma membrane is required for the recruitment of clathrin adaptors and other endocytic factors to this membrane, while the tight coupling of $PI(4,5)P_2$ dephosphorylation by synaptojanin to the dynamin-dependent fission reaction of endocytosis is required to allow the shedding of such factors once the vesicle has undergone separation from the plasma membrane (Cremona *et al.*, 1999; Cremona and De Camilli, 2001; Wenk *et al.*, 2001). We also discovered curvature-generating/sensing proteins (BAR domain–containing proteins, primarily endophilin) that are responsible for achieving this coupling by coordinating the formation of the narrow neck of endocytic buds with the recruitment of synaptojanin (Takei *et al.*, 1999; Farsad *et al.*, 2001; Frost *et al.*, 2009; Wu *et al.*, 2010; Milosevic *et al.*, 2011). This model, which is encapsulated in Figure 1 and which is supported by genetic studies in mice and other model organisms, posits that a cycle of PI phosphorylation and dephosphorylation is nested within the synaptic vesicle cycle. The implication of a phosphoinositide phosphatase in membrane transport converged with the identification of a PI kinase (the PI 3-kinase Vps34) involved in "vacuolar protein sorting" in yeast (Schu *et al.*, 1993). This raised the possibility that the phosphorylation and dephosphorylation of inositol phospholipids could have a general significance in membrane traffic (De Camilli *et al.*, 1996).

The field grew very rapidly. It was found that reversible phosphorylation of phosphatidylinositol at the 3,4 and 5 positions of the inositol ring by a multiplicity of enzymes generates seven phosphoinositide species whose differential protein-binding specificities help control, often via a dual key mechanism (Wenk and De Camilli, 2004), membrane cytosol interfaces (Di Paolo and De Camilli, 2006; Vicinanza *et al.*, 2008; Balla, 2013). Along with small GTPases (Behnia and Munro, 2005), phosphoinositides are key determinants of the identity of membranes or membrane subdomains and thus control the multiplicity of reactions that occur at membrane–cytosol interfaces, such as the recruitment and shedding of trafficking proteins, the function of integral membrane proteins, and the assembly and disassembly of signaling and cytoskeleton complexes. Importantly, it became clear that, as we had found for synaptojanin, interconversion of phosphoinositide species along the secretory and endocytic pathways functions as a switch to release membrane-associated factors that define one compartment and to recruit factors that define the next compartment (Odorizzi *et al.*, 2000; Di Paolo and De Camilli, 2006; Zoncu *et al.*, 2009). I think I would not have immediately grasped the significance of the identification of synaptojanin and then invested much of our work in this field had it not been for my early research experience in medical school.

The roles of phosphoinositides in membrane identity imply the existence of mechanisms to tightly control their localizations and also to ensure availability of PI in membranes—primarily the plasma membrane—where the backbone of this lipid is consumed by PLC. Surprisingly, genetic evidence suggested that one mechanism to control PI4P levels in membranes is via their direct contacts with the endoplasmic reticulum (ER), where a main PI4P phosphatase is localized (Foti *et al.*, 2001; Stefan *et al.*, 2011; Mesmin *et al.*, 2013). Concerning availability of PI, DAG generated by PLC and its phosphorylated downstream product phosphatidic acid must be returned to the ER for metabolic recycling by the ER-localized phosphatidylinositol synthase, and newly synthesized PI then needs to be rapidly transported to the plasma membrane to replace its depleted pool. Strong evidence indicates that these reactions are mediated at least in part by lipid transfer proteins, and we have now learned that much of this protein-mediated lipid transport occurs at membrane

contact sites (Mesmin *et al.*, 2013; Wong *et al.*, 2017; Cockcroft and Raghu, 2018; Pemberton *et al.*, 2020). This is what motivated my lab to enter the young field of membrane contact sites. Here, once again, my early appreciation of the importance of lipid transfer proteins during medical school had an impact on my decision to invest in this rapidly developing area of cell biology.

Not only has it been rewarding to help expand the inventory of proteins known to function both as membrane tethers and as lipid transporters, and to elucidate their function and regulation (Giordano *et al.*, 2013; Schauder *et al.*, 2014; Chung *et al.*, 2015; Dong *et al.*, 2016; Saheki *et al.*, 2016), but this has been an opportunity, in collaboration with my Yale colleague Karin Reinisch, to participate in the discovery of something unexpected and new in the biology of eukaryotic cells. Until recently, it was thought that nonvesicular lipid transport, including the transport occurring at membrane contact sites, occurred only via protein modules that function as shuttles between two bilayers, often acting as countertransporters, delivering different cargoes as they move back and forth between two closely apposed membranes. However, our investigations of VPS13, a protein originally identified in yeast for its function in membrane transport and subsequently linked to lipid dynamics (Lang *et al.*, 2015; Park *et al.*, 2016), raised the possibility that this protein, and thus also the closely related autophagy factor ATG2, may be the founding members of a protein superfamily with bulk lipid transport properties, that is, proteins that could facilitate the net fluxes of lipids between adjacent membranes, and thus in membrane expansion (Kumar *et al.*, 2018). As was subsequently shown by structural studies (Valverde *et al.*, 2019; Li *et al.*, 2020), these rod-like proteins harbor a hydrophobic groove that spans their length, thus supporting a bridge-like model of lipid transport to support bulk lipid flow (Li *et al.*, 2020; Guillen-Samander *et al.*, 2021; Leonzino *et al.*, 2021). Inspection of protein sequence and structure databases suggests the existence of several other proteins with these properties, implying that this mode of lipid transport (a bridge-like mechanism), previously described for the transport of lipids from the inner to the outer membrane of Gram-negative bacteria (Bishop, 2019), may be of broad relevance in cell biology. As mutations in VPS13 family proteins result in neurodegenerative diseases, including a Huntington-like disease and Parkinson's disease (Ugur *et al.*, 2020), this is a field rich in medical implications. In fact, my curiosity about disease mechanisms stemming from my medical school training contributed to making me specially interested in studying these proteins.

It has been a wonderful journey, rich with twists and turns, exploring new territories and continuously discovering that there are untapped frontiers whose existence we still do not know about. During the course of my career, I found especially rewarding bringing together different fields and finding new connections between fundamental cell biology and medicine, more so in recent years, where genetic studies of diseases often provide the first clues to molecular and cellular mechanisms. As I have emphasized in this essay, each of our experiences, no matter how successful, becomes part of the body of knowledge that defines us and has an impact on our research trajectory. I typically encourage students to search for training opportunities in different settings rather than follow a linear path, as this will enable them to bring together in their independent careers the expertise and knowledge of different worlds and thus to carry out original science. Our specific and unique paths are what make our research innovative and special.

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